



Detection of mutant infectious bronchitis viruses of GI-23 lineage from commercial chicken flocks in Khorasan Razavi province, Iran in 2019

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ABSTRACT

Infectious bronchitis (IB), caused by infectious bronchitis virus (IBV), is one of the most important respiratory diseases in poultry. The implementation of preventive measures, including vaccination and biosecurity, is necessary for controlling the disease. To maintain biosecurity, it is important to identify the entry route of new viruses into a region and characterizing markers such as unique mutations that make viruses traceable. During a genotyping study for IBV infected commercial chicken flocks in Khorasan Razavi province, 11 viruses from 11 broiler and layer chicken flocks were detected in different cities by PCR. Sequencing of the S1 partial gene followed by phylogenetic analysis showed that eight viruses can be classified in GI-23 lineage (Is-Variant2), two viruses are classified in GI-1 lineage (Mass), and one virus is classified in GI-12 lineage (793B). Although detected viruses of GI-23 lineage are originated from Iran, seven viruses have synonymous (T954C and G1056A) and non-synonymous (C797T) mutations that have not been previously reported. It was found that the new genetic changes in Iranian IBVs of GI-23 lineage occurred in two different regions in Khorasan Razavi. In conclusion, this study indicates that the high prevalence of GI-23 lineage viruses in Iran may enhance the chance of virus mutations and the emergence of new viral strains, so effective vaccination and biosecurity measures are required to control the virus spread.

Keywords

IBV, genotyping, GI-23 lineage, mutation, Iran

Abbreviations

IBV: infectious bronchitis virus
IB: infectious bronchitis
PCR: polymerase chain reaction

GI: genotype I
Mass: Massachusetts

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Introduction

Infectious bronchitis virus (IBV) causes a respiratory disease called avian infectious bronchitis (IB) that was first reported in the 1930s in the United States [1]. IB is highly contagious and causes severe economic losses in the poultry industry worldwide [2]. All strains of IBV can replicate in the respiratory tract of birds and cause respiratory diseases. Some IBV strains can also target epithelial cells in the oviducts and kidneys and may cause a significant reduction in egg production, nephritis, and mortality [2]. The pathogenicity of different strains of IBV can be categorized from mild respiratory involvement to severe kidney disease [3].

IBV has a single-stranded, positive-sense, RNA genome approximately 27 kb in length. The 3' end of the genome encodes four non-structural proteins, including 3a, 3b, 5a, and 5b, as well as four structural proteins, including the glycoprotein spike (S), envelope (E), membrane (M), and nucleocapsid (N). The 5' end of the genome encodes two polypeptides (1a and 1ab) that are required for RNA amplification [4]. Genetic variation in IBV can occur following recombination or mutations such as deletion, insertion, and substitution during virus replication [2].

The high rate of mutation in the genome of coronaviruses is associated with the poor ability of enzymatic correction of mutations (3' to 5' exonuclease activity) during replication [5]. The spike protein is composed of about 1145 amino acids and is cleaved into two subunits, S1 and S2, following post-translational modifications [6]. The S1 protein determines the virus serotype and contains the neutralizing epitopes. Considering the IBV genetic variation and the highest diversity of the S1 gene, the emergence of new serotypes and genotypes is expectable [6]. The high rate of changes has led to the emergence of new IBV genotypes in different parts of the world [7]. According to the latest IBV genotyping method by using the S1 gene, six genotypes are defined that comprise 32 distinct viral lineages and some inter-lineage recombinants [7]. Genotype I (GI) includes 27 lineages and each of the remaining five genotypes contains 1 lineage. Lineages such as GI-1 (Mass type), GI-13 (793B type), GI-19 (LX4 or QX), GI-16 (Q1), GI-21 (Italy02), and GI-23 (Israel variant 2), have been reported in several continents, countries, or regions, while other lineages are limited to specific regions of the world [7].

The first report of IBV infection in chicken flocks in Iran was published in 1994 [8]. Since then, the IBV prevalence in Iran has been reported by several studies [9-11]. In the latest study in Khorasan Razavi province in 2016, GI-23 lineage viruses were common and had a 100% genetic similarity to other Iranian IBVs [12].

GI-23 lineage viruses have also the highest prevalence in Iran [9, 10].

As in previous studies reported from Khorasan Razavi and Iran [9, 10, 12], we found that GI-23 lineage viruses are prevalent in Khorasan Razavi, with the difference that Is-Variant2 viruses detected in this study have point mutations that distinguish them from previously reported viruses. This finding emphasizes that effective vaccination and biosecurity programs are needed to prevent the emergence and spread of new IBV strains. The viruses detected in this study will be traceable due to specific mutations and will make it possible to track outbreak routes of a virus in different regions.

Results

IBV detected in 11 commercial chicken flocks by PCR and sequencing

Among the 15 IB suspected commercial broiler and layer chicken flocks in Khorasan Razavi, 11 flocks belonging to the cities of Mashhad (5 flocks), Torbat Heydariyeh (2 flocks), Quchan (1 flock), Gonabad (1 flock), Kadkan (1 flock), and Chenaran (1 flock) were IBV positive.

Prevalence of GI-23 lineage viruses with synonymous and non-synonymous mutations in the Khorasan Razavi province

The partial sequences of the S1 gene of positive samples were submitted to GenBank, and accession numbers MW366335 to MW366345 were assigned to the 11 detected viruses. The nucleotide sequences of these viruses, along with reference strains and some Iranian IBVs were used for phylogenetic analysis. Out of 11 viruses, eight viruses detected in Mashhad, Torbat Heydariyeh, Gonabad, Quchan, Kadkan, and Chenaran were classified as GI-23 lineage (Israel Variant 2). From three strains identified in Mashhad two were classified as GI-1 lineage (Mass type) and one as GI-13 lineage (793B type) (Figure 1).

Four GI-23 viruses identified in Mashhad (MW366340 and MW366344), Chenaran (MW366345), and Quchan (MW366342) formed a distinct subbranch (60% bootstrap support) and are 100% similar in nucleic acid sequence (Figures 1 and 2). These DNA sequences BLASTed against GenBank (December 2020). The highest similarity (99.38%) was related to 8 viruses. These viruses include IBV/Chicken/Iran/IS1494-like/MRB02/2016 (MG013973), IBV/Chicken/Iran/IS1494-like/MRB01/2016 (MG013972), Iran/Bu/Variant2/SH1229.7/14 (KX578827), Iran/variant 2/H272/12 (KP310024), SEMNAN/18/2018 (MN794044), SEMNAN/10/2018 (MN794036), SEM-

NAN/5/2018 (MN794031) and SEMNAN/4/2018 (MN794030), all detected in Iran during the 2010s. Synonymous point mutations including T954C and G1056A were identified in the S1 gene compared to the previously reported Iranian IBVs.

The three viruses identified in the cities of Torbat Heydariyeh (MW366335 and MW366336) and Gonabad (MW366341) were also classified in the GI-23 lineage and formed a distinct subbranch (65% bootstrap support) (Figures 1 and 2). These viruses are 100% similar in nucleic acid sequence. Following BLASTing the nucleotide sequences against GenBank (December 2020), the highest similarity (99.7%) was related to 11 viruses. These viruses include IBV/Chicken/Iran/IS1494-like/MRB02/2016 (MG013973), IBV/Chicken/Iran/IS1494-like/MRB01/2016 (MG013972), Iran/Bu/Variant2/SH1450.19/15 (KX578834), Iran/Bu/Variant2/SH1450.5/15 (KX578832), Iran/Bu/Variant2/SH1229.7/14 (KX578827), Iran/variant 2/H272/12 (KP310024), Iran/Variant 2/H100/11 (KP310023), SEMNAN/18/2018 (MN794044), SEMNAN/10/2018 (MN794036), SEMNAN/5/2018 (MN794031) and SEMNAN/4/2018 (MN794030), all identified in Iran during the 2010s. A non-synonymous point mutation C797T that results in an amino acid exchange of Thr266Met was identified in the S1 gene compared to the previously reported Iranian IBVs.

The virus (MW366343) was identified in Kadkan city was clustered along with other Iranian GI-23 lineage viruses (Figure 1). This virus shows 100% similarity with the most previously reported Iranian IBVs of the GI-23 lineage.

The virus (MW366338) detected in Mashhad was classified in GI-11 (Figure 1). Nucleotide analysis showed that this virus is 100% similar to the 793B vaccine virus.

Based on the phylogenetic tree, two IBVs which were identified in Mashhad (MW366337 and MW366339) are classified as GI-1. However, these two viruses, with 100% similarity, formed a distinct subbranch (67% bootstrap support) (Figure 1). The nucleotide sequences BLASTed against GenBank (December 2020), the highest similarity (100%) was related to 3 viruses including Iran/Mass/H350/12 (KP310052), ck/CH/LJL/121059/2012 (KJ425509), and China/vaccine strain/HK/2004 (AY761141). Analysis of nucleotide similarities showed that the nucleotide sequences of these two viruses are 99.68%, 99.36%, and 98.73% similar to Ma5, H120, and H52 vaccine viruses, respectively; however, the protein sequences are 100% similar.

Phylogenetic analysis based on amino acid sequences of GI-23 lineage viruses

The deduced amino acid sequences of GI-23

lineage viruses corresponding to partial S1 protein used for the phylogenetic tree construction (Figure 3). The 5 viruses detected in Mashhad (MW366340 and MW366344), Chenaran (MW366345), Quchan (MW366342), and Kadkan (MW366343) were clustered along with other GI-23 lineage viruses reported from Iran. The protein sequences of these 5 viruses are 100% similar to the most Iranian GI-23 viruses previously reported. The amino acid sequences BLASTed against GenBank (December 2020). The highest similarity (100%) was related to 4 viruses including SEMNAN/3/2018 (QLF98578), Iran/Bu/Variant2 /SH1229.7/14 (ART85681), SEMNAN/10/2018 (QLF98585), and Iran/variant 2/H272/12 (AKH60829).

The viruses detected in Torbat Heydariyeh (MW366335 and MW366336) and Gonabad (MW366341) are 100% similar in amino acid sequence and form a distinct subbranch (65% bootstrap support) (Figure 3). For further investigation, the amino acid sequences BLASTed against GenBank (December 2020). The most similar viruses were 99.1% similar and included 7 viruses Iran/Bu/Variant2/SH1450.5/15 (ART85686), Iran/Bu/Variant2/SH1450.19/15 (ART85688), SEMNAN/3/2018 (QLF98578), Iran/Variant 2/H100/11 (AKH60828), Iran/Bu/Variant2/SH1229.7/14 (ART85681), SEMNAN/10/2018 (QLF98585) and Iran/variant 2/H272/12 (AKH60829).

Discussion

The GI-23 lineage represents a cluster of unique wild-type viruses that are geographically limited to the Middle East. Strains belonging to this lineage have been identified in Israel since 1998 and are still circulating in the region [10, 13]. Some of these viruses like Is-Variant2 have become prevalent and affect the respiratory and renal systems [14]. Studies in recent years have shown that variant 2 viruses are common in Iran [9, 10].

Among the GI-23 lineage IBVs identified in this study, the virus detected in the city of Kadkan (MW366343) has 100% nucleotide similarity with the viruses reported in the Khorasan Razavi province in 2016 [12] and other GI-23 lineage viruses previously reported in Iran [9]. Accordingly, this virus is currently present from the previous outbreaks of GI-23 lineage viruses in Iran.

The other seven GI-23 lineage viruses of this study have point mutations different from other viruses reported in GenBank. These viruses formed two distinct subbranches, and each subbranch contained similar viruses that were detected in adjacent cities (Figures 1 and 2).

Nucleotide BLAST showed that there are no re-

ported viruses with 100% similarity to GI-23 lineage viruses detected in Mashhad (MW366340 and MW366344), Chenaran (MW366345), and Quchan (MW366342). However, the closest sequences (8 viruses) with 99.38% similarity have been reported from Iran during the 2010s, and all differed from the viruses of this study in two bases, 954 and 1056. Nevertheless, the amino acid sequence is 100% similar to the Iranian GI-23 viruses. Accordingly, unique synonymous point mutations make the virus traceable in future studies. It should be noted that the virus had

the chance to spread to adjacent cities (Figure 2).

The other GI-23 lineage IBVs detected in Torbat Heydarieh (MW366335 and MW366336) and Gonabad (MW366341) are 100% similar (Figures 1 and 2). Nucleotide and protein BLAST showed that the sequence with 100% similarity was not registered in GenBank. The most similar sequences to these viruses have been reported from Iran; so, these viruses also originated in Iran. However, a non-synonymous point mutation (C797T) has been created in the detected viruses compared to the previously reported ones.

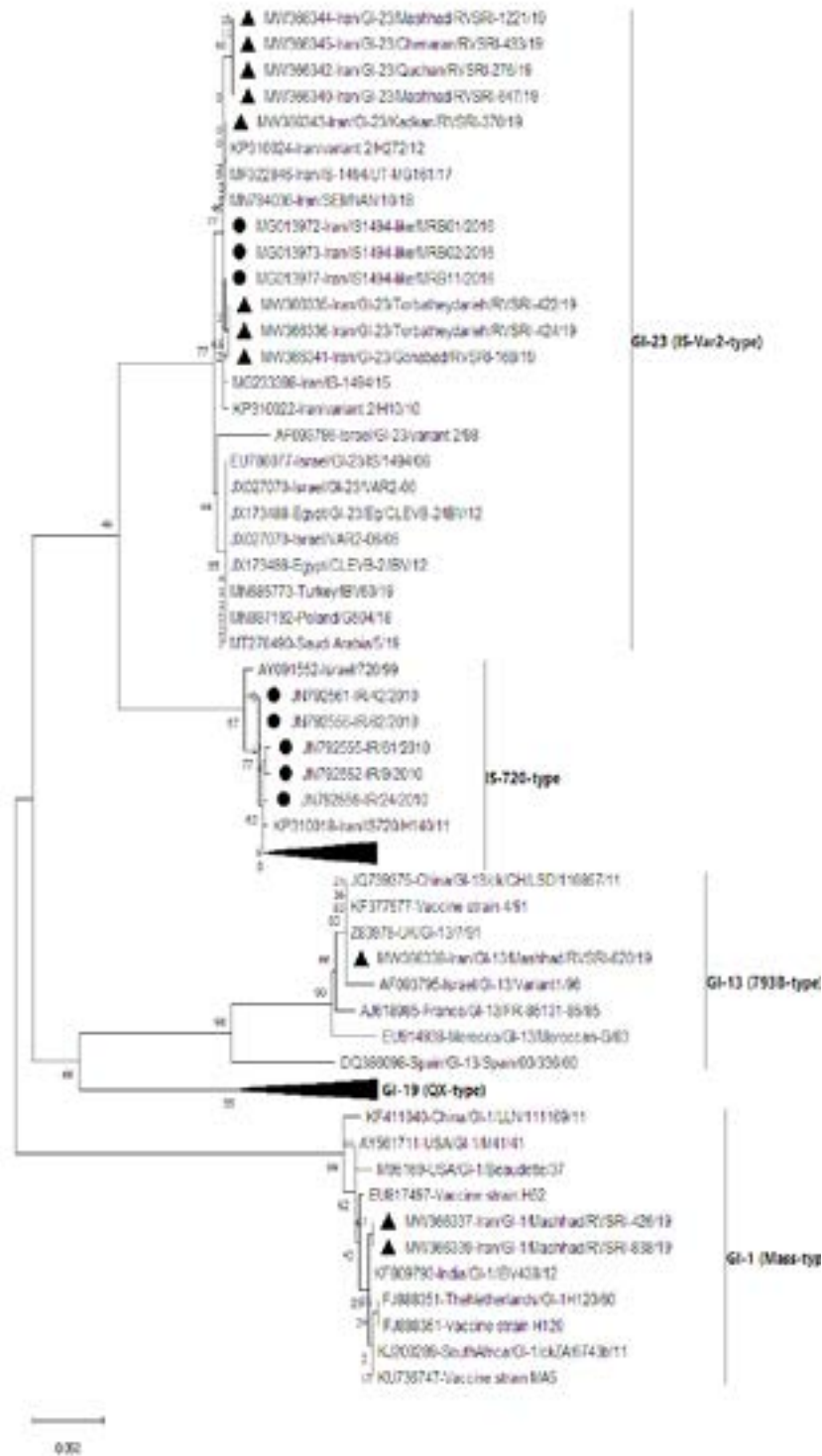


Figure 1. Maximum Likelihood phylogenetic tree of the partial nucleotide sequences of the S1 gene. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model with 1000 bootstrap replicates. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 70 nucleotide sequences. There was a total of 314 positions in the final dataset. IBV strains previously identified in the Khorasan Razavi province are indicated by a filled circle. IBV strains identified in this study are indicated by a filled triangle. Evolutionary analyses were conducted in MEGA X.

Materials & Methods

Sampling

15 commercial broiler and layer flocks with clinical signs and gross lesions suspected of IBV infection located in the Khorasan Razavi province were examined for IBV genotypes in 2019. The vaccination program in these flocks was mostly in the form of the Massachusetts vaccines and in some cases as a combination of Massachusetts and 793B vaccines. During necropsy, sampling of tracheal, renal, and cecal tonsil tissues was performed from 5 to 10 birds of each flock. After transferring tissue samples to the laboratory on the ice pack, the samples were stored at -70°C. Molecular tests were performed in the Research Department laboratory of Razi Vaccine and Serum Research Institute (RVSRI), Mashhad, Iran.

Viral RNA isolation

RNA isolation was performed using the High Pure Viral RNA Kit (Roche). The quality and quantity of the isolated genomic RNA were evaluated using NanoDrop 2000c spectrophotometer. Some of the isolated RNA was directly used to make cDNA, and the remaining RNA was stored at -70°C.

This mutation that caused amino acid changes in the S1 protein, results in the formation of a distinct subbranch in the phylogenetic tree of protein sequences (Figure 3). It is noteworthy that this virus also had the chance to spread to adjacent cities (Figure 2).

In conclusion, this study shows that although the detected viruses originated in Iran, probably two IBVs with unreported point mutations have spread in two separate regions in the Khorasan Razavi province. Given that in one of these viruses detected in Torbat Heydarieh and Gonabad, a non-synonymous point mutation resulted in a change in the S1 protein, the virus may have different virulence and neutralizing epitopes than other GI-23 lineage viruses previously reported in Iran. Based on the information of this study, the possible prevalence of these viruses can be traced in future studies in Iran, and to improve biosecurity measures, possible routes of virus entry into different regions can be identified.

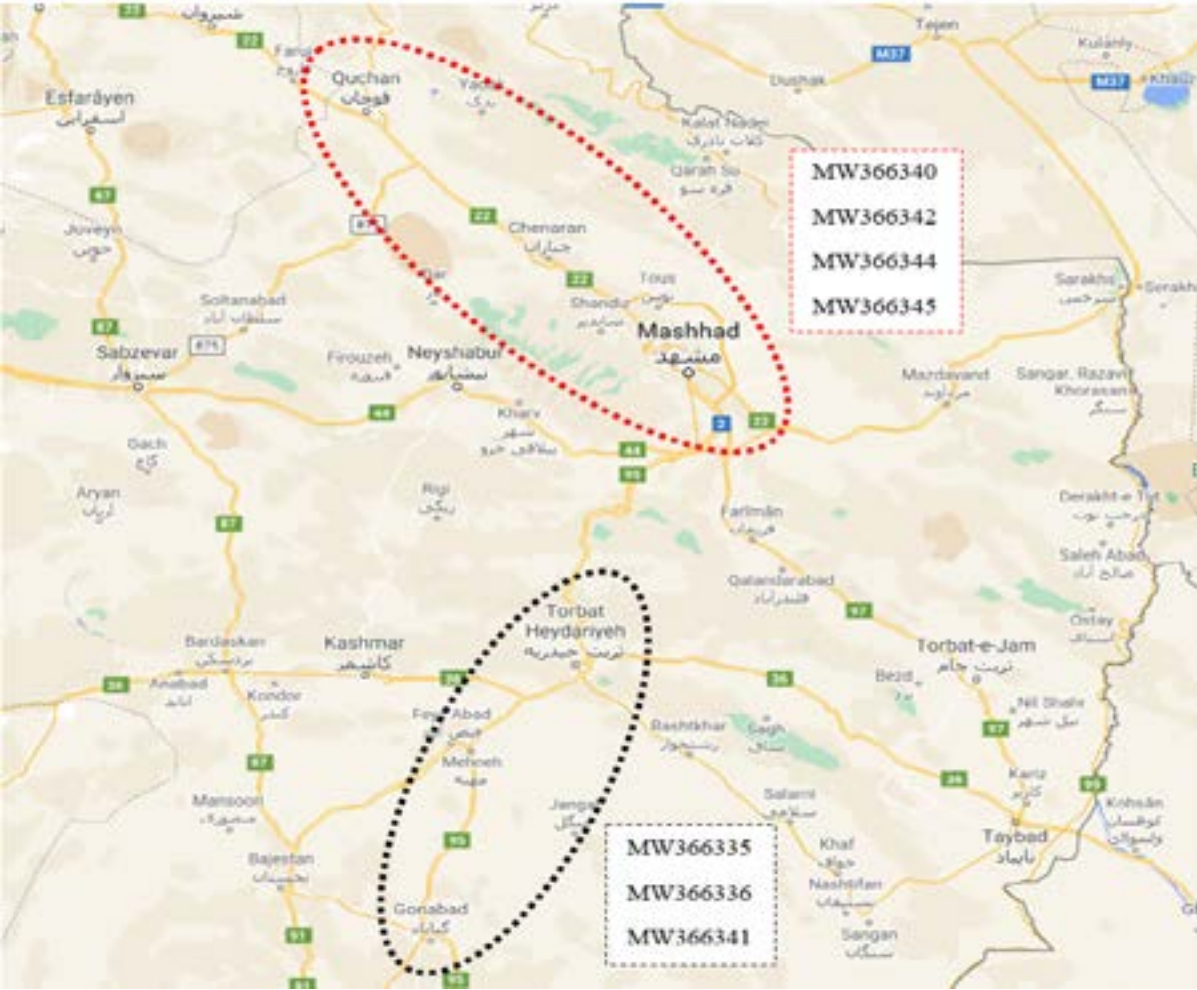


Figure 2. Map of the prevalence of mutated IBV strains in two regions in the Khorasan Razavi province. Viruses with 100% similarity have been identified in the areas shown inside an ellipse. The virus GenBank accession numbers are listed next to each ellipse. Google map image from: <https://www.google.com/maps/@35.9244275,58.3266594,8z>

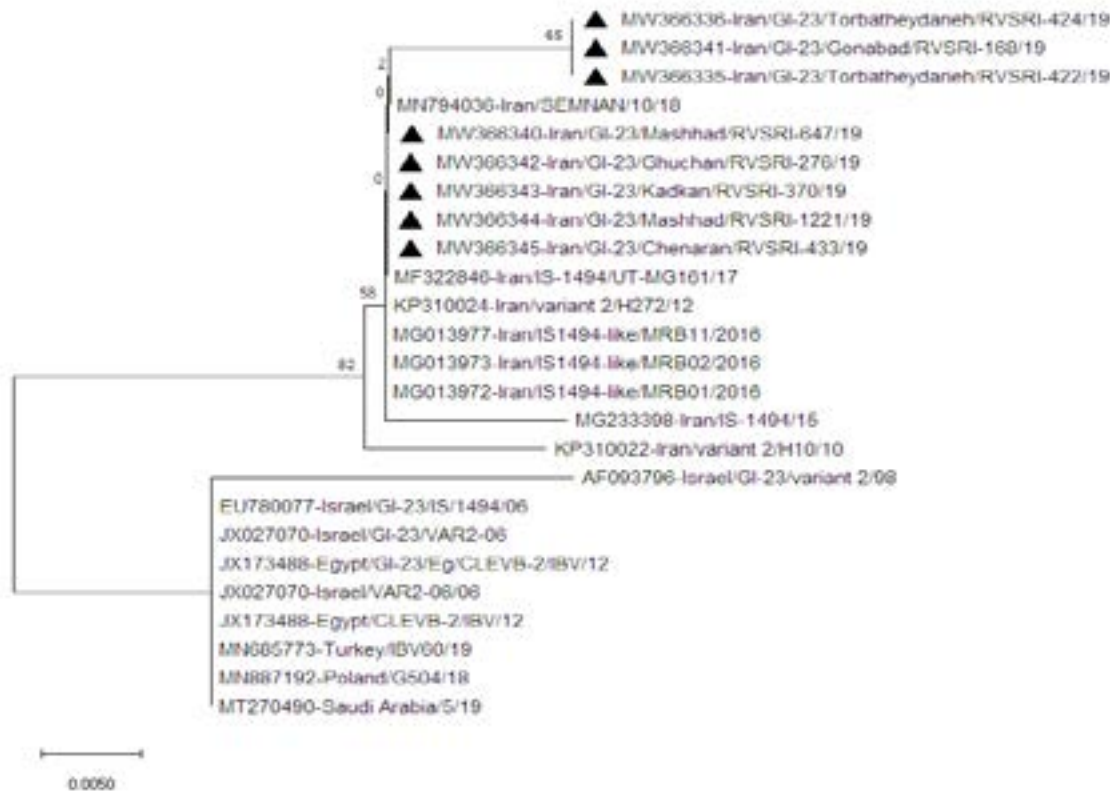


Figure 3. Maximum Likelihood phylogenetic tree of the S1 partial gene amino acid sequences of GI-23 lineage viruses. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model with 1000 bootstrap replicates. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 25 amino acid sequences. There was a total of 107 positions in the final dataset. IBV strains identified in this study are indicated by a filled triangle. Evolutionary analyses were conducted in MEGA X.

Reverse Transcription (RT) Reaction

cDNA synthesis was performed using M-MuLV reverse transcriptase (RevertAid, Thermo Scientific, Carlsbad, CA, USA) immediately after RNA isolation. A specific primer of the S1 partial gene called SX2 was used to make cDNA (Table 1) [15]. The cDNA was employed for PCR and the remainder was stored at -20 °C.

PCR and nested PCR

PCR and nested PCR were performed using primers XCE1, SX2, SX3, and SX4 (Table 1) [15, 16]. These primers are common to most known strains of IBV and amplify a segment of the S1 gene that varies between IBV genotypes. PCR and nested PCR were performed in a total volume of 20 µl using a mixture of Taq polymerase 2X Master Mix (Ampliqon, Odense, Denmark), 1 µl (10 µM) of each forward and reverse primer, 1 µl of the template DNA, and 7 µl of sterile distilled water. Amplification was performed with a thermal profile (1 step of 95°C for 5 min, 35 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec, and 1 step of 72°C for 5 min) for PCR and nested PCR. For the first PCR, XCE1 and SX2 primers were used and the second PCR (nested PCR) was performed using the first PCR product (one microliter of one to ten dilution) as template and SX3 and SX4 primers. For all PCR steps, the negative control reaction consisted sterile distilled water instead of the template DNA. H120 and 793B vaccine viruses were used for positive control reactions to verify the

performance of cDNA synthesis and PCR reactions.

Evaluation of nested PCR reactions and nucleotide sequencing

To evaluate the nested PCR for the presence of 393 bp band, gel electrophoresis was performed with 1.5% agarose. PCR products were sequenced (Bioneer, Korea) by using specific primers. The chromatograms obtained from the sequencing were examined and the results were edited as needed. Confirmed results were used for phylogenetic analysis.

Phylogenetic analysis of nucleotide and amino acid sequences

For phylogenetic analysis, the variable region sequences of the S1 gene related to 27 viruses with defined genotypes [7], were taken from GenBank (<http://www.ncbi.nlm.nih.gov>). By searching in GenBank, S1 gene sequences of 20 IBV strains related to the Khorasan Razavi province isolated from 2010 to 2016 were downloaded. Genetically similar sequences with strains detected in this study, based on BLAST results, included strains from Iran and other countries, were also obtained from GenBank for tree construction. Nucleotide alignment of these sequences was performed by ClustalW algorithm implemented in BioEdit software version 7.5.2 [17]. Phylogenetic analysis was performed using partial sequences of S1 genes in ME-

GA-X software [18]. After analyzing to find the best models for tree construction, the Maximum Likelihood statistical method, GTR + G substitution models, and test of phylogeny by bootstrap method with 1000 replications were used. Due to the importance of studying amino acid changes in teh S1 pro-

tein, phylogenetic tree construction was performed based on partial S1 amino acid sequences of GI-23 lineage viruses. After analyzing to find the best models for tree construction, the Maximum Likelihood statistical method, JTT substitution model, and test of phylogeny by bootstrap method with 1000 replications were employed.

Table 1. Sequence and position of the oligonucleotide primers used in PCR and nested PCR

Oligonucleotide	Sequence (5' - 3')	Position in S1 sequence	Reference
XCE1 +	CACTGGTAATTTTTCAGATGG	728 to 749	16
SX2 -	TCCACCTCTATAAACACCYTT	1148 to 1168	15
SX3 +	TAATACTGGYAATTTTTCAGA	705 to 725	15
SX4 -	AATACAGATTGCTTACAACCACC	1075 to 1097	15

Authors' Contributions

S-E.T., R.T. and H.F. conceived and planned the experiments. S-E.T., R.T., M.S., M.F. and E.V. carried out the experiments. S-E.T. carried out the nucleotide and phylogenetic analysis. R.T., N.M.A., S.S., J.A.A., M.G., M.T., M.F., N.K., M.K.A. M.J.M., A.S., and T.M. contributed to sample preparation. S-E.T. contributed to the interpretation of the results. S-E.T. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research and analysis. All authors have read and approved the final draft of the manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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